

Identification of the N-Terminal Functional Domains of Cdk5 by Molecular Truncation and Computer Modeling

Jianwen Zhang,¹ Chi-Hao Luan,² Kuo-Chen Chou,³ and Gail V.W. Johnson^{1*}

¹Department of Psychiatry, University of Alabama at Birmingham, Birmingham, Alabama

²Center for Biophysical Sciences & Engineering, University of Alabama at Birmingham, Birmingham, Alabama

³Computer-Aided Drug Discovery, Upjohn, Kalamazoo, Michigan

ABSTRACT Cyclin dependent kinase (Cdk) 5, an atypical member of the Cdk family, plays a fundamental role in the development of the nervous system, and may also be involved in the pathogenesis of certain neurodegenerative diseases. Further, Cdk5 is activated by the specific regulatory proteins p39, p35, or p25 rather than cyclins, and in contrast to other members of the Cdk family is not involved in the progression of the cell cycle. A three-dimensional computer model of Cdk5-p25-ATP has been generated previously [Chou et al., *Biochem Biophys Res Commun* 1999;259:420–428], providing a structural basis for the study of the mechanisms of Cdk5 activation. To assess the predicted ATP and p25 binding domains at the N-terminal of Cdk5, two mutants of Cdk5 were prepared in which amino acids 9–15 (Δ 9–15) or 9–47 (Δ 9–47) were deleted. The results of these studies clearly demonstrate that an N-terminal loop and the PSSALRE helix are indispensable for Cdk5-p25 interactions, and amino acids 9–15 are necessary for ATP binding but are not involved in Cdk5-p25 interactions. Predicted models of Δ 9–15 Cdk5 and Δ 9–47 Cdk5 were generated, and were used to interpret the experimental data. The experimental and molecular modeling results confirm and extend specific aspects of the original predicted computer model, and may provide useful information for the design of highly selective inhibitors of Cdk5, which could be used in the treatment of certain neurodegenerative conditions. *Proteins* 2002; 48:447–453. © 2002 Wiley-Liss, Inc.

Key words: cdk5; p25; p35; protein phosphorylation; molecular modeling

INTRODUCTION

Based on sequence homology, cyclin-dependent kinase (Cdk) 5 is a member of the Cdk family.¹ However, Cdk5 is a unique member of this family in that it is active in postmitotic neurons,¹ and is activated by the regulatory proteins p39, p35, or p25 rather than cyclins.^{2–4} p39 and p35 are unique gene products and are highly enriched in the nervous system.⁵ p25 is a 208-residue C-terminal proteolytic product of p35.^{6,7} The domains of p35 involved in interacting with and activating Cdk5 reside entirely within the p25 sequence,⁸ and therefore p35 and p25 would be predicted to similarly activate Cdk5. However, p25 is much longer lived in the cell than p35, possibly due

to decreased proteasomal degradation resulting from the loss of a ubiquitination signal.^{9,10} In addition, p25 has been reported to show a differential distribution within the cell compared to p35.¹⁰ Therefore, although p35 and p25 regulate Cdk5 in a similar manner, the differential distribution and increased stability of p25 could result in an increase in Cdk5 activation by p25 in the cell compared to p35.

Cdk5 itself is widely expressed. However, Cdk5 activity is detected almost exclusively in the nervous system due to the selective localization of p35 and p39.^{1,8} Cdk5 has been shown to play a pivotal role in neuronal development and differentiation,⁸ and there is also evidence that a dysregulation of Cdk5 (possibly due to increased levels of p25) may contribute to the pathological processes in Alzheimer's disease brain.¹⁰ Further, there is data suggesting that aberrant activation of Cdk5 may play a role in facilitating apoptosis.^{10–14}

The activity of Cdk is tightly regulated and dependent upon association with p39 or p35/p25,^{2–4} although certain phosphorylation events may also modulate its activity.^{15,16} Cdk5 shows high sequence identity to Cdk2. However, there is little homology between the cyclins and p39 or p35/p25, the regulatory subunits of Cdk5.^{2–4} Using different molecular approaches, several studies have elucidated the specific domains of p35 that are important for its interaction with, and activation of, Cdk5.^{17–20} Further, these earlier studies indicated that the activation domain of p35/p25 may adopt a cyclin-fold structure,¹⁸ which has recently been verified by a crystal structure analysis of Cdk5-p25.²¹

To better understand the interaction between Cdk5 and p25 and the mechanisms by which Cdk5 is activated, three-dimensional computer modeling of Cdk5/p25²² has been carried out based on the X-ray structure of the Cdk2-cyclin A-ATP complex^{23,24} and the predicted cyclin-fold structure within p35/p25.¹⁸ This model, which is in

Grant sponsor: NIH; Grant number: NS35060; Grant sponsor: NASA; Grant number: NCC8-126.

Jianwen Zhang's current address is Biocryst Pharmaceuticals, Inc., 2190 Parkway Lake Drive, Birmingham, AL 35244.

*Correspondence to: Dr. Gail V.W. Johnson, Department of Psychiatry and Behavioral Neurobiology, University of Alabama, at Birmingham, Birmingham, AL 35294-0017. E-mail: gvwj@uab.edu

Received 1 January 2002; Accepted 28 March 2002

accordance with the recently published crystal structure of Cdk5/p25,²¹ provided the initial structural basis for this study on the mechanisms of Cdk5 activation. Although the crystal structure data elucidated the role of the T-loop in regulating Cdk5 activity,²¹ the role of the N-terminal region of Cdk5 in the activation process was not fully described. In addition, the Cdk5-p25 crystal structure was determined in the absence of ATP. Therefore, to assess the predicted ATP and p25 binding domains at the N-terminal of Cdk5, two N-terminal deletion mutants of Cdk5 were made in which amino acids 9–15 ($\Delta 9$ –15) or 9–47 ($\Delta 9$ –47) were deleted. These constructs, as well as wild type Cdk5, were used in kinase and co-immunoprecipitation assays to determine the role of these particular domains in ATP and p25 binding. Further, molecular modeling of Cdk5 and its two deletion mutants, $\Delta 9$ –15 and $\Delta 9$ –47, in complex with p25 and ATP, was also carried out. The experimental results of these studies clearly demonstrate that an N-terminal loop and the PSSALRE helix are indispensable for Cdk5-p25 interactions, and amino acids 9–15 are necessary for catalytic activity, but are not involved in Cdk5-p25 interactions. These results thus confirm and extend specific aspects of the predicted three-dimensional computer model of the Cdk5-p25-ATP complex²² and are consistent with the structural analysis by the molecular modeling in this study.

MATERIALS AND METHODS

Constructs

The CMV expression vectors for p25 and Cdk5 were generous gifts from Dr. Tsai at Harvard University.^{3,10} The Cdk5 insert was subcloned into the BamH1 site of pcDNA3.1 (+), and then used as the template for PCR to generate deletion constructs of Cdk5. To prepare the two N-terminal deletion constructs of Cdk5 ($\Delta 9$ –47 and $\Delta 9$ –15) based on the predicted functional domains of Cdk5,^{6,9} five synthetic primers, P1, 5'-CAAGTGTATCATATGCCAATACGC-3'; P2, 5'-ATAGTTAGCGGC-CGCTTCCAGTTTCTCGTATTTCTCGT-3'; P3, 5'-ATAAGAAATGCGGCCGCCCTCCGGGAGATCTGC-3'; P4, 5'-CAGGGATCCCTAGGGCGGACAGAAGTCGG-3' and P5, 5'-ATAAGAATGCGGCCGCCGGAAGTGTGTTCAAG-GCCA-3' were used in the following combinations. For the construct of $\Delta 9$ –47 mutant, Cdk5/pcDNA3.1 (+) was used as a template, and the P1 (forward) and P2 (reverse) primers were used to produce the 1.2 kb F1 fragment with a 5' Nde1 site from the vector and a 3' Not1 site. The 750-bp F2 fragment was generated using the P3 (forward) and P4 (reverse) primers, which started with the codon for Ala⁴⁸ of Cdk5 with a 5' Not1 site and ended with the stop codon for Cdk5 and a 3' BamH1 site. After PCR, the products were purified and digested with Nde1 and Not1 for F1 and with Not1 and BamH1 for F2. After gel purification the fragment F1 and F2 were ligated into pcDNA3.1 (+), which was digested with Nde1 and BamH1. To generate the $\Delta 9$ –15 mutant of Cdk5, the F1 fragment was prepared as described above, and the F3 fragment was prepared by PCR using the P5 (forward) and P4 (reverse) primers. F3 is an 850-bp

fragment with a 5' Not1 site followed by the codon for Gly¹⁶ of Cdk5 through the codon for the last amino acid of Cdk5 followed by BamH1 site. After digestion and purification, the F1 and F3 fragments were ligated into pcDNA3.1(+) as described above. In both mutants ($\Delta 9$ –47 and $\Delta 9$ –15), the deleted sequences were replaced with two Ala. After preparation all constructs were verified by sequencing.

Cell Culture and Transient Co-Transfection

CHO cells were grown in 100-mm Corning plates in F12 media supplemented with 5% Fetalclone II (HyClone), 20 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies). When the cells were approximately 80% confluent, they were co-transfected with the constructs p25/Cdk5, p25/ $\Delta 9$ –15 or p25/ $\Delta 9$ –47 using Fugene 6 (Roche) in serum-free media. Twenty-four hours later, the transfected cells were harvested, washed with cold PBS, and stored at -80°C until use.

Cell Lysis and Immunoprecipitation

Cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 2.5 mM MgCl_2 , 0.14 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 10 μ g/ml aprotinin, 0.5 μ M okadaic acid, 100 μ M β -glycerophosphate. Lysates were clarified by centrifugation at 20,000g at 4°C for 30 min, and protein concentrations were determined. Extracts (500 μ g) were incubated with either 2 μ g Cdk5 polyclonal antibody (C-8, Santa Cruz) or 2 μ g of p35 polyclonal antibody (C-19, Santa Cruz) (which also recognizes p25) at 4°C for 1 h prior to the addition of 50 μ l of prewashed protein A Sepharose 6B (Pharmacia) beads, and incubation with end-to-end rotation at 4°C overnight. After incubation, the beads were washed three times with lysis buffer containing 0.25 M NaCl and once with lysis buffer. The washed precipitates were used for immunoblotting and kinase assays.

Immunoblotting

Immunoblot analysis was carried out as described previously.¹³ In brief, cell lysates were run on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to nitrocellulose, and probed with the p35 polyclonal antibody, the Cdk5 monoclonal antibody (DC17, Santa Cruz) or the Cdk5 polyclonal antibody. Immunoblots were quantified using a Bio-Rad GS670 imaging densitometer.

To detect the binding between p25 and Cdk5, $\Delta 9$ –15 or $\Delta 9$ –47, the immunoprecipitated beads were boiled in a $2 \times$ SDS Stop buffer for 5 min, the beads were pelleted, and the immunoprecipitated proteins subjected to SDS-PAGE, followed by immunoblotting with either the Cdk5 polyclonal antibody or the p35 polyclonal antibody. Protein A conjugated HRP (Bio-Rad) was used instead of goat anti-rabbit IgG-HRP to detect the presence of the proteins.

Kinase Assay

To measure kinase activity, 50 μ l of immunoprecipitated beads were resuspended in 100 μ l of kinase buffer contain-

ing 25 mM Tris-HCl, pH7.4, 10 mM MgCl₂, 0.5 mM (dithiothreitol) DTT, 5% glycerol, 18 µg histone H1 (Calbiochem). The kinase reactions were initiated by adding 50 µM ATP, 5 µCi [γ -³²P] ATP (Amersham Pharmacia Biotech), and incubated at 30°C for the time points indicated. To stop the reaction, 10 µl of the mixture was transferred to a microfuge tube containing 10 µl of 2 × SDS Stop buffer and boiled for 3 min. The phosphorylated histone H1 was resolved by electrophoresis on a 10% SDS-polyacrylamide gel, and analyzed using phosphoimaging (Molecular Dynamics) as described previously.¹³ For quantitatively analyzing the kinase activity, the phosphorylation data from phosphoimaging were normalized to the p25 levels in the cell lysates as determined by immunoblotting.

Molecular Modeling

Homology modeling was carried out to obtain structural information for the three molecular complexes, Cdk5-p25-ATP, Cdk5(Δ9–15)-p25-ATP, and Cdk5(Δ9–47)-p25-ATP. Exploiting Cdk5's high homology (70%) and high identity (60%) to Cdk2,¹ a three-dimensional structure for Cdk5 was generated using the web-based *SWISS-MODEL Protein Modelling Server*.²⁵ The structure yielded was from a number of X-ray structures of Cdk2 in complex with cyclin A and ATP, including that of Jeffery et al.²⁴ [pdb code 1FIN]. The Cdk5-p25-ATP structure thus obtained was highly homologous to the Cdk2 structure in complex with cyclin A and ATP. The *SWISS-MODEL* automated process, however, failed to produce a structure for p25 even though the program was instructed to use the cyclin A X-ray structure in [1FIN] as template because of low-sequence homology. Therefore, a model for p25 was created by aligning residues 141–291 of p25 (numbering is based on the sequence of p35 because p25 is a proteolytic product of this protein) with residues 171–320 of cyclin A as described previously.^{18,22} Using this alignment, the backbone structure of p25 was derived from that of cyclin A as described by Jeffery et al.²⁴ The side chain conformations were determined by several cycles of 20 picosecond molecular dynamics simulated annealing at 600 or 300 K followed by slow-cooling stage and energy minimization. The backbone atoms were fixed in the first few cycles of the refinement. The constraints were gradually relaxed to harmonic constraints with force constant varied for different regions of the molecule where residues in regions connecting the α -helices were subjected to weaker constraint than that in regions of the conserved helices. The molecular dynamics simulation was performed using both Cartesian coordinates and torsion angles. In addition to molecular dynamics at elevated temperature, a dielectric constant of 80 was also used in energy minimization to perturb conformations in local minima. To obtain a model for the Cdk5-p25-ATP complex, the modeled structures of the three components were superimposed on the X-ray structure of Cdk2-cyclin A-ATP complex²⁴ by best fitting the N-terminal lobe of Cdk5 to that of Cdk2 and the helices of p25 to that of cyclin A. The same refinement strategy

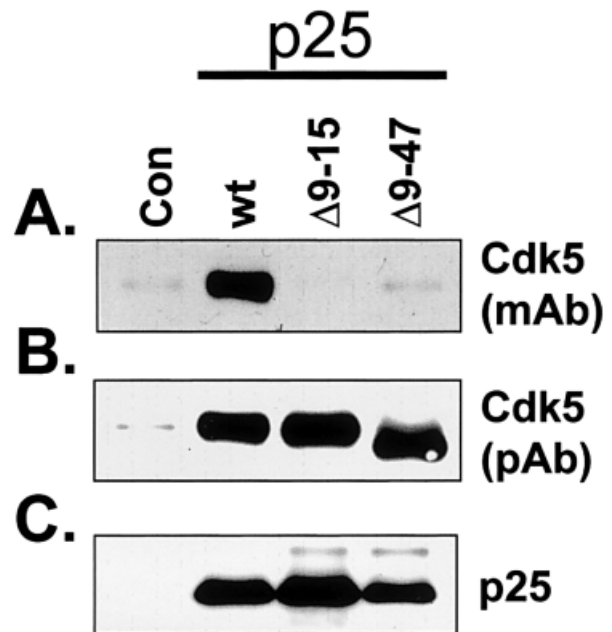


Fig. 1. Expression levels of Cdk5, Cdk5 Δ9–15 or Cdk5 Δ9–47 and p25 in co-transfected CHO cell lysates. CHO cells were co-transfected with the constructs p25/Cdk5, p25/Δ9–15 or p25/Δ9–47. Twenty-four hours post transfection, the cells were harvested, and cell lysates (25 µg protein/lane) were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with the Cdk5 monoclonal antibody (A), the Cdk5 polyclonal antibody (B), or the p35 polyclonal antibody, which also recognizes p25 (C).

described above was applied to the model of Cdk5-p25-ATP complex with emphasis on optimizing the residues involved in intermolecular interaction for which the harmonic constraints applied were less restrictive than for residues remote from the intermolecular interfaces. The modeling was carried out using the software *Crystallography and NMR System (CNS)*²⁶ and its force field on the Cray SV1 supercomputer in the Alabama Supercomputer Center. The model structures of Cdk5 and p25 were then superimposed on the X-ray structure of the Cdk2-cyclinA-ATP complex²⁴ by best-fitting only the N-terminal lobe of Cdk5, and subjected to energy minimization with proper constraints. The model (depicted in Fig. 4) is quite similar to that described by Chou et al.²² This structure served as the template for the two Cdk5 mutants in complex with p25 and ATP.

In Δ9–15 Cdk5, removal of residues 9–15 and the addition of two Ala residues results in the sequence M¹QKYEKLEAAG, instead of the wild type Cdk5 sequence, M¹QKYEKLEKIGEGTYG. Modeling for Δ9–15 Cdk5 was started by filling the backbone coordinates of residues 6–15 in the Cdk5 structure into the 10-residue sequence MQKYEKLEAA, followed by energy minimization with proper constraints. A similar procedure was applied to the Δ9–47 Cdk5 mutant. The molecular modeling was concentrated on the structural analysis of the consequences of these truncations on the binding capacity of Cdk5 for ATP and p25.

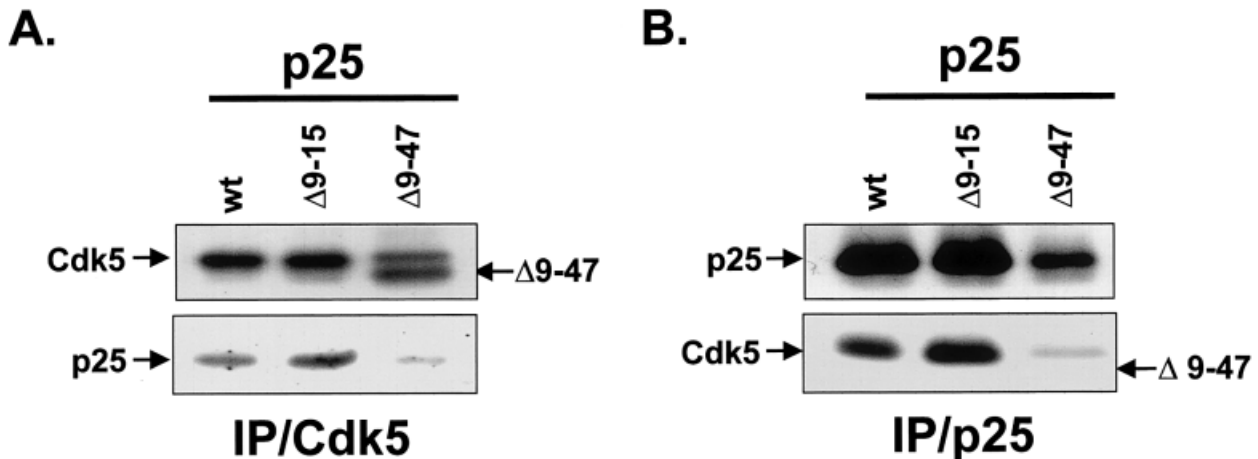


Fig. 2. Identification of the binding between Cdk5 and p25. Cells were transfected with p25 and the indicated Cdk5 construct and then precipitated with either the Cdk5 polyclonal antibody (A) or the p25 polyclonal antibody (B), and the resulting precipitates were probed for both Cdk5 and p25. The polyclonal Cdk5 antibody precipitated all the Cdk5 constructs. In the lane showing the precipitation of the $\Delta 9-47$ construct, the upper band in the Cdk5 blot (A, top panel) is endogenous Cdk5. p25 co-precipitated with both wild type and $\Delta 9-15$ Cdk5. However, almost no p25 was found in the $\Delta 9-47$ precipitate; the p25 that is present likely is due to interaction with endogenous Cdk5 (A, bottom panel). Wild type and $\Delta 9-15$ Cdk5 co-precipitated with p25. However, $\Delta 9-47$ Cdk5 did not co-precipitate (B), although endogenous Cdk5 did come down with p25 (B, bottom panel, far right lane).

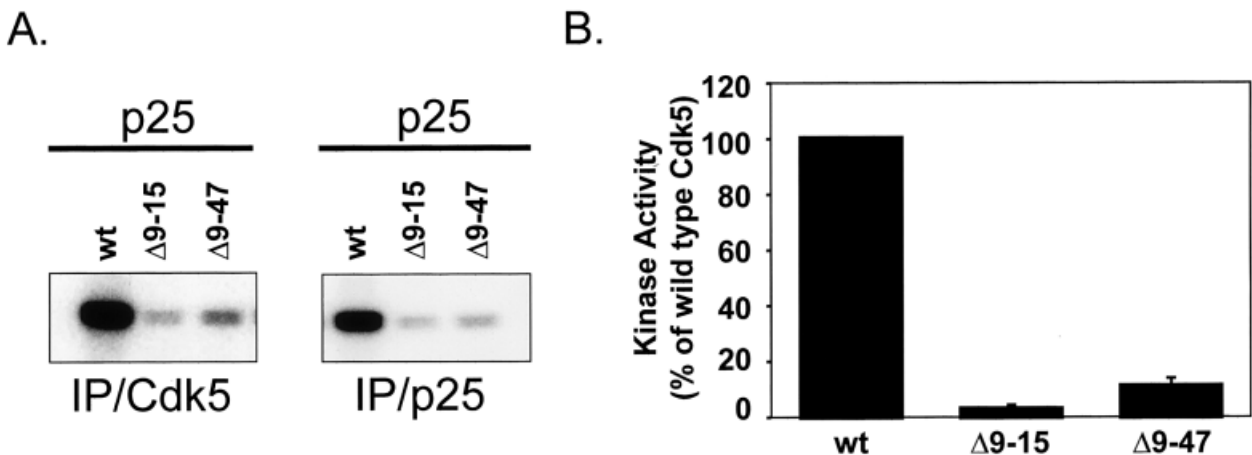


Fig. 3. Comparison of the kinase activity of Cdk5, $\Delta 9-15$ Cdk5, and $\Delta 9-47$ Cdk5. Cells were transfected with p25 and the indicated Cdk5 construct, precipitated with either the Cdk5 polyclonal (left) or the p25 polyclonal antibody (right) and used in a kinase assay with histone H1 as the substrate. The reaction was terminated after 10 min, the phosphorylated histone H1 was resolved by SDS-PAGE and analyzed using phosphorimaging. Representative data is shown in (A). For quantitative analyzing the kinase activity, the results from the phosphorimaging were normalized to the p25 levels in the cell lysates as determined by immunoblotting. The data in (B) are from 3 separate experiments, and activity is presented as a percent of wild type (wt) Cdk5 activity.

RESULTS AND DISCUSSION

Expression of p25 With Either Cdk5, $\Delta 9-15$, or $\Delta 9-47$

To identify the N-terminal domains of Cdk5 that are important for catalytic activity and p25 binding, two deletion mutants of Cdk5 were constructed based on a predicted three-dimensional model of the Cdk5-ATP-p25 complex.²² In $\Delta 9-15$, amino acids 9–15 of Cdk5, which contains a predicted ATP binding site,²² were replaced by two Ala encoded by the Not1 restriction site that was introduced into the Cdk5 insert. In $\Delta 9-47$, amino acids 9–47, which contain a potential ATP binding site, an N-loop, and a small portion of the PSSALRE helix, a pseudo p25 interaction domain based on homology between Cdk5 and other Cdk family members,²² were de-

leted and replaced by two Ala due to the introduction of the Not1 site into the Cdk5 insert. Cells were co-transfected with p25 and Cdk5, $\Delta 9-15$ or $\Delta 9-47$, and protein expression was examined by immunoblotting. CHO cells express endogenous Cdk5 [see faint band in control (Con) lane of Fig. 1(A) and (B)]. However, transfection with wild type Cdk5 results in a robust increase in expression that is detected by both monoclonal and polyclonal Cdk5 antibodies [Fig. 1(A) and (B)]. In contrast, the expression of $\Delta 9-15$ and $\Delta 9-47$ was not detected by the monoclonal Cdk5 antibody [Fig. 1(A)]. The inability of the monoclonal Cdk5 antibody to recognize the deletion constructs of Cdk5 is likely due to the fact that this antibody recognizes an N-terminal sequence that has been deleted or is disrupted

in the mutants. For the $\Delta 9-47$ mutant, an increase in mobility is observed due to the removal of 36 amino acids [Fig. 1(B)]. No endogenous expression of p25 or p35 was observed in the CHO cell. However, co-transfection of p25 with all of the Cdk5 constructs resulted in robust expression of the protein [Fig. 1(C)].

Amino Acids 16–47 in the N-Terminal Region of Cdk5 Are Essential for p25 Binding

Co-immunoprecipitation was used to determine the interaction of Cdk5 and the mutants with p25. Cells were

co-transfected with p25 and Cdk5, $\Delta 9-47$ or $\Delta 9-15$. The cell lysates were immunoprecipitated with the polyclonal Cdk5 antibody and probed for the presence of Cdk5 and p25. Both wild type and $\Delta 9-15$ mutant Cdk5 co-precipitated p25. However, $\Delta 9-47$ did not, even though it was precipitated by the Cdk5 antibody [Fig. 2(A)]. The small amount of p25 in the $\Delta 9-47$ precipitates [Fig. 2(A), bottom panel] is due to interaction with the endogenous Cdk5 [Fig. 2(A), top panel]. To confirm these results, cells were again transfected with p25 and Cdk5, $\Delta 9-47$ or $\Delta 9-15$, and then immunoprecipitated with the p35 antibody and probed for the presence of Cdk5 and p25. These results confirmed that $\Delta 9-47$ mutant Cdk5 did not bind p25 [Fig. 2(B)], although endogenous Cdk5 did co-precipitate with p25 in the $\Delta 9-47$ transfected cells [Fig. 2(B), right lane, bottom panel]. These data demonstrate that amino acids 16 to 47 of Cdk5, which contain a portion of PSSARLE sequence and an N-terminal loop immediately preceding the PSSARLE sequence, form an essential p25 binding domain. However, amino acids from 9–15 of Cdk5 are not involved in p25 binding. The importance of PSTAIRE helix of Cdk2 in cyclin binding has been demonstrated previously,²⁴ and the PSSALRE helix in Cdk5 adopts an identical position²¹ and thus is essential for the interaction of p25 (p35) with Cdk5. Interestingly, the N-loop of Cdk5 is essentially a short sequence mainly composed of five acidic amino acids (DDDDE), which is predicted to directly interact with α -helix 6 of p25 (p35) by strong salt bridges in addition of hydrogen bonds.²² In contrast, even though the N-loop fragment (DTETE) of Cdk2 is also acidic, two Asp residues are replaced with two neutral amino acids (Thr) in comparison with the N-loop of Cdk5.



Fig. 4. The schematic drawing of a computer-modeled structure for the Cdk5-p25-ATP complex. The Cdk5 backbone is yellow, p25 is red, and ATP as a space-filling molecule in the CPK color scheme. This figure was produced using the program *Ribbons*.³¹

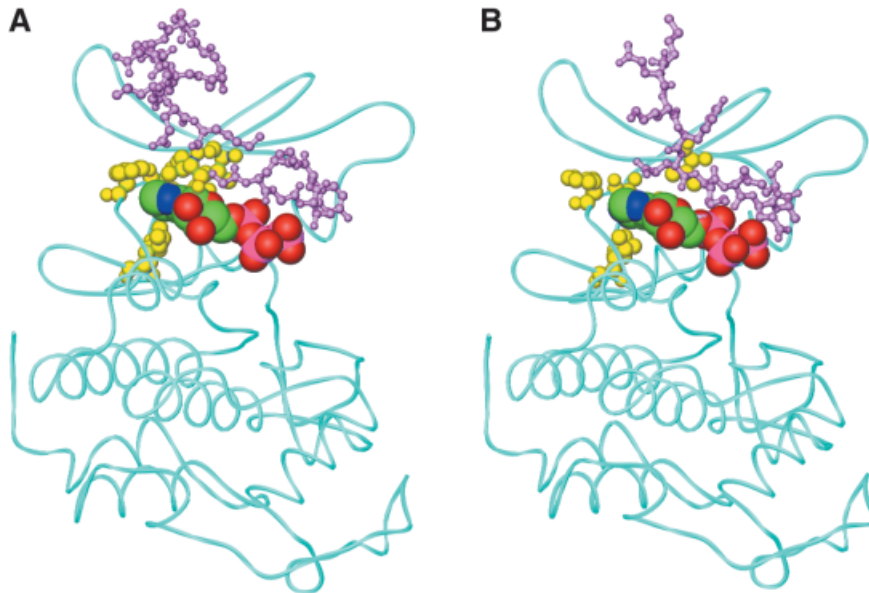


Fig. 5. Computer-modeled structures of the Cdk5-p25-ATP complex (A) and the Cdk5 ($\Delta 9-15$)-p25-ATP complex (B). The amino terminus residues relevant to the truncation ($\Delta 9-15$) are shown in purple with side chains. The C-terminal residues of Cdk5 are shown in the backbone trace in blue. ATP is shown as a space-filling molecule in CPK color. The hydrophobic residues Ile¹⁰ (absent in Cdk5 (9–15)), Val¹⁸, Leu⁶⁴, Phe⁸⁰, Phe⁸², Leu¹³³ forming the hydrophobic half-pocket are shown in yellow with side chains. These figures were produced using the program *Ribbons*.³¹

Thus, the salt bridge interactions between the N-loop of Cdk2 and cyclin A are likely weaker than those occurring between the N-loop of Cdk5 and p25.²² This may result in a significant difference in the affinity and interaction of Cdk2 and Cdk5 with their respective partners.

Deletion of 9–15 Abolishes the Kinase Activity of Cdk5

To determine the effects of the deletions on the catalytic activity of Cdk5, the complexes of Cdk5/p25, Δ9–15/p25 and Δ9–47/p25 were immunoprecipitated with either the Cdk5 or p35 polyclonal antibody, and used to measure kinase activity. As expected wild type Cdk5 was fully activated when co-transfecting together with p25. However, Δ9–15 showed essentially no activity, and Δ9–47 showed low activity [Fig. 3(A)]. When kinase activity was normalized to the levels of p25 in the corresponding cell lysates, the activity of Δ9–47 was 11% of the wild type Cdk5, while Δ9–15 was 2.5% and represented just background activity [Fig. 3(B)]. Given the finding that Δ9–47 does not bind p25 and the fact that there is endogenous Cdk5 in CHO cells, the activity in the Δ9–47 precipitates is likely due to endogenous Cdk5 activity [Fig. 3(B)]. In contrast, the Δ9–15 mutant still retains its p25 binding capability and thus prevents endogenous Cdk5 from binding the p25 and, therefore, acts as a dominant negative mutant.

Consistency With the Computer-Modeled Structure

As with other Cdk proteins, the predicted structure of Cdk5,²² consists of two lobes: the N-terminal lobe (residues 1–85), which is composed of a 5-strand β-sheet and the conserved PSSALRE helix, and the C-terminal lobe (residue 86–291), which is composed mostly by α-helices and the T-loop, shown in Figure 4. The interaction between Cdk5 and p25 (p35) is predicted to occur mainly in the T-loop region and N-terminal lobe of Cdk5 that includes the PSSALRE helix and an N-terminal loop located immediately preceding the PSSALRE segment of Cdk5. Therefore, deletion of the N-loop and first 3 amino acids of the PSSALRE helix would be predicted to disrupt the binding between Cdk5 and p25. This was observed experimentally with the Δ9–47 mutant.

The ATP binding pocket is located between the two lobes of Cdk5. Based on a previous model,²² the following amino acid residues of Cdk5 are predicted to form the ATP binding pocket: Ile¹⁰, Gly¹¹, Glu¹², Gly¹³, Thr¹⁴, Val¹⁸, Ala³¹, Val⁶⁴, Phe⁸⁰, Glu⁸¹, Phe⁸², Cys⁸³, Asp⁸⁴, Asp⁸⁶, Lys⁸⁹, Lys¹²⁸, Gln¹³⁰, Asn¹³¹, Leu¹³³, Ala¹⁴³, and Asp¹⁴⁴. These residues form two important characteristics of the ATP binding pocket: a hydrophobic half-cage for the adenine and ribose ring, and a GXGXXG motif for the phosphates of ATP. The side chains of Ile¹⁰, Val¹⁸, Leu⁶⁴, Phe⁸⁰, Phe⁸², Leu¹³³ are oriented as such to form a hydrophobic half-cage for the adenine and ribose ring of ATP, as shown in Figure 5(A). The 17-residue amino terminal sequence, M¹Q²K³Y⁴E⁵K⁶L⁷E⁸K⁹I¹⁰G¹¹E¹²G¹³T¹⁴Y¹⁵G¹⁶T¹⁷, of Cdk5 contains the most highly conserved sequence motif in protein kinases for ATP binding, GXGXXG, also known as a glycine rich motif.^{27–29}

Deletion of residues 9–15, K⁹I¹⁰G¹¹E¹²G¹³T¹⁴Y¹⁵, affected both of these critical features for ATP binding. From the structural perspective, K⁹I¹⁰G¹¹E¹²G¹³T¹⁴Y¹⁵ forms the first β-strand and the loop in the five β-strands of the N-terminal lobe of Cdk5. In the Δ9–15 construct, the sequence MQKYEKLEAA, i.e., the first 8 residues in Cdk5 plus two Ala residues, becomes the N-terminal sequence, replacing the 15 residue sequence in Cdk5. The possibility for this new amino terminus sequence to potentially fill in the structural positions of residues 9–15 of the Cdk5 structure was explored and no plausible arrangement was found that could keep the hydrophobic and polar characteristics of the portion of the ATP binding pocket. For instance, when the sequence, EKLEAA, replaced I¹⁰G¹¹E¹²G¹³T¹⁴Y¹⁵ in the Cdk5 structure, the resulting change to the ATP binding pocket is the loss of Ile¹⁰ as replaced by a Glu in the hydrophobic portion and the loss of the GXGXXG motif, especially with the two key Gly residues replaced by a Lys and a Glu residue. As shown in Figure 5(B), unfavorable interaction between the new sequence and the other part of the 5-strand β-sheet would distort the β-strand conformation and disrupt the ATP binding pocket, even if the other parts of the protein structure were kept unchanged.

Therefore, it is clear that the 9–15 deletion changed the ATP binding pocket by affecting the hydrophobic half-cage and altered the GXGXXG motif due to the change from IGEITYG to EKLEAAG. This would predict that when the amino acids between 9–15 in Cdk5 were deleted, its ATP binding capability should be affected. This would offer an explanation for the loss of catalytic activity in the Cdk5 (Δ9–15) mutant (although ATP binding was not measured directly). Based on these findings, it can be predicted that point mutations at Ile¹⁰, Gly¹¹, or Gly¹³ should also eliminate Cdk5's ATP binding capability. However, amino acids 9–15 are not predicted to be a component of the N-terminal binding domain of Cdk5, and no significant impact on the interaction between Cdk5 and p25 was observed experimentally when residues 9 to 15 were deleted.

The deletion of residues 9 to 47, however, has a great effect on the N-terminal portion of Cdk5 structure. The 5-strand β-sheet is the main portion of the N-terminal lobe of the Cdk5 structure. The 9–47 deletion left only the residues in two strands of β-sheets. This would greatly reduce the possibility of these residues forming the β-sheet conformation to interact effectively with p25. This plus the truncation of the stretch of acidic residues before the PSSALRE helix would destroy the interaction of Cdk5 with p25, as was observed in the experimental results.

In conclusion, the experimental and modeling data as reported in the present study have elucidated several aspects of the original predicted three-dimensional structure of Cdk5²² and extended these original findings providing a clearer picture of the mechanisms by which Cdk5 is regulated. Further, there is data to suggest that Cdk5 may play a role in certain neurodegenerative diseases,^{9,30} and therefore Cdk5 may provide a potentially important therapeutic target. The molecular modeling and experimental

data presented in this study clearly indicate which residues and domains are essential for Cdk5-p25(p35) interactions. This information could be very useful in developing specific inhibitors of Cdk5 using structure-based drug design.

ACKNOWLEDGMENTS

This work was supported by NIH grant NS35060 (G.V.W.J.) and by NASA's cooperative agreement NCC8-126 to the Center for Biophysics and Engineering, UAB (C.H.L.). The authors thank the Alabama Supercomputer Center for use of its hardware and software systems and Dr. Tsai (Harvard University) for providing the Cdk5 and p25 constructs.

REFERENCES

- Meyerson M, Enders GH, Wu CL, Su LK, Gorka C, Nelson C, Harlow E, Tsai LH. A family of human cdc2-related protein kinases. *Embo J* 1992;11:2909–2917.
- Tang D, Yeung J, Lee K Y, Matsushita M, Matsui H, Tomizawa K, Hatase O, Wang J H. An isoform of the neuronal cyclin-dependent kinase 5 (Cdk5) activator. *J Biol Chem* 1995;270:26897–26903.
- Tsai LH, Delalle I, Caviness VS Jr, Chae T, Harlow E. p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* 1994;371:419–423.
- Lew J, Huang QQ, Qi Z, Winkfein RJ, Aebersold R, Hunt T, Wang JH. A brain-specific activator of cyclin-dependent kinase 5. *Nature* 1994;371:423–426.
- Smith DS, Greer PL, Tsai LH. Cdk5 on the brain. *Cell Growth Differ* 2001;12:277–283.
- Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH. Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature* 2000;405:360–364.
- Kusakawa G, Saito T, Onuki R, Ishiguro K, Kishimoto T, Hisanaga S. Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. *J Biol Chem* 2000;275:17166–17172.
- Dhavan R, Tsai LH. A decade of cdk5. *Nat Rev Mol Cell Biol* 2001;2:749–759.
- Patrick GN, Zhou P, Kwon YT, Howley PM, Tsai LH. p35, the neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5) is degraded by the ubiquitin-proteasome pathway. *J Biol Chem* 1998;273:24057–24064.
- Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* 1999;402:615–622.
- Ahuja HS, Zhu Y, Zakeri Z. Association of cyclin-dependent kinase 5 and its activator p35 with apoptotic cell death. *Dev Genet* 1997;21:258–267.
- Gao C, Negash S, Wang HS, Ledee D, Guo H, Russell P, Zelenka P. Cdk5 mediates changes in morphology and promotes apoptosis of astrocytoma cells in response to heat shock. *J Cell Sci* 2001;114:1145–1153.
- Zhang J, Johnson GV. Tau protein is hyperphosphorylated in a site-specific manner in apoptotic neuronal PC12 cells. *J Neurochem* 2000;75:2346–2357.
- Zhang Q, Ahuja HS, Zakeri ZF, Wolgemuth DJ. Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling. *Dev Biol* 1997;183:222–233.
- Zukerberg LR, Patrick GN, Nikolic M, Humbert S, Wu CL, Lanier LM, Gertler FB, Vidal M, Van Etten RA, Tsai LH. Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. *Neuron* 2000;26:633–646.
- Sharma P, Sharma M, Amin ND, Albers RW, Pant HC. Regulation of cyclin-dependent kinase 5 catalytic activity by phosphorylation. *Proc Natl Acad Sci USA* 1999;96:11156–11160.
- Lim HY, Seow KT, Li Q, Kesuma D, Wang JH, Qi RZ. Structural Insights into Cdk5 activation by a neuronal Cdk5 activator. *Biochem Biophys Res Commun* 2001;285:77–83.
- Tang D, Chun AC, Zhang M, Wang JH. Cyclin-dependent kinase 5 (Cdk5) activation domain of neuronal Cdk5 activator. Evidence of the existence of cyclin fold in neuronal Cdk5a activator. *J Biol Chem* 1997;272:12318–12327.
- Wang X, Ching YP, Lam WH, Qi Z, Zhang M, Wang JH. Identification of a common protein association region in the neuronal Cdk5 activator. *J Biol Chem* 2000;275:31763–31769.
- Poon RY, Lew J, Hunter T. Identification of functional domains in the neuronal Cdk5 activator protein. *J Biol Chem* 1997;272:5703–5708.
- Tarricone C, Dhavan R, Peng J, Areces LB, Tsai L, Musacchio A. Structure and regulation of the cdk5-p25(nck5a) complex. *Mol Cell* 2001;8:657–669.
- Chou KC, Watenpaugh KD, Heinrikson RL. A model of the complex between cyclin-dependent kinase 5 and the activation domain of neuronal Cdk5 activator. *Biochem Biophys Res Commun* 1999;259:420–428.
- De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgan DO, Kim SH. Crystal structure of cyclin-dependent kinase 2. *Nature* 1993;363:595–602.
- Jeffrey PD, Russo AA, Polyak K, Gibbs E, Hurwitz J, Massague J, Pavletich NP. Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* 1995;376:313–320.
- Guex N, Diemand A, Peitsch MC. Protein modelling for all. *Trends Biochem Sci* 1999;24:364–367.
- Brunger AT, Adams PD, Clore GM, Delano WL, Gros P, Grosse-Kunstleve RW, Jiang J-S, Kusewski J, Nilges N, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography and NMR system (CNS): A new software system for macromolecular structure determination. *Acta Cryst* 1998;D54:905–921.
- Hanks SK, Quinn AM, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* 1988;241:42–52.
- Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *Faseb J* 1995;9:576–596.
- Hemmer W, McGlone M, Tsigelny I, Taylor SS. Role of the glycine triad in the ATP-binding site of cAMP-dependent protein kinase. *J Biol Chem* 1997;272:16946–16954.
- Nguyen MD, Lariviere RC, Julien JP. Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. *Neuron* 2001;30:135–147.
- Carson M. Ribbons. *Methods Enzymol* 1997;277:493–505.